The Plant Cell, Vol. 11, 1897–1909, October 1999, www.plantcell.org

ABI1 Protein Phosphatase 2C Is a Negative Regulator of Abscisic Acid Signaling

Françoise Gosti,[a,1] Nathalie Beaudoin,[a] Carine Serizet,[a] Alex A. R. Webb,[b,2] Nicole Vartanian,[a] and Jérôme Giraudat[a,3]

[a] Institut des Sciences Végétales, Centre National de la Recherche Scientifique UPR 40, avenue de la Terrasse, 91190 Gif-sur-Yvette, France

[b] Department of Biological Sciences, Lancaster University, Bailrigg, Lancaster LA1 4YQ, United Kingdom

The plant hormone abscisic acid (ABA) is a key regulator of seed maturation and germination and mediates adaptive responses to environmental stress. In Arabidopsis, the *ABI1* gene encodes a member of the 2C class of protein serine/threonine phosphatases (PP2C), and the *abi1-1* mutation markedly reduces ABA responsiveness in both seeds and vegetative tissues. However, this mutation is dominant and has been the only mutant allele available for the *ABI1* gene. Hence, it remained unclear whether *ABI1* contributes to ABA signaling, and in case *ABI1* does regulate ABA responsiveness, whether it is a positive or negative regulator of ABA action. In this study, we isolated seven novel alleles of the *ABI1* gene as intragenic revertants of the *abi1-1* mutant. In contrast to the ABA-resistant *abi1-1* mutant, these revertants were more sensitive than the wild type to the inhibition of seed germination and seedling root growth by applied ABA. They also displayed increases in seed dormancy and drought adaptive responses that are indicative of a higher responsiveness to endogenous ABA. The revertant alleles were recessive to the wild-type *ABI1* allele in enhancing ABA sensitivity, indicating that this ABA-supersensitive phenotype results from a loss of function in ABI1. The seven suppressor mutations are missense mutations in conserved regions of the PP2C domain of ABI1, and each of the corresponding revertant alleles encodes an ABI1 protein that lacked any detectable PP2C activity in an in vitro enzymatic assay. These results indicate that a loss of ABI1 PP2C activity leads to an enhanced responsiveness to ABA. Thus, the wild-type ABI1 phosphatase is a negative regulator of ABA responses.

INTRODUCTION

Abscisic acid (ABA) plays a major role in various aspects of plant growth and development, including seed maturation and germination, as well as adaptation to abiotic environmental stresses (Zeevaart and Creelman, 1988; McCarty, 1995; Rock and Quatrano, 1995; Leung and Giraudat, 1998). Although the nature of the ABA receptor(s) remains unknown, substantial progress has been made recently in the characterization of more downstream elements of the ABA signaling pathways (Busk and Pagès, 1997; Bonetta and McCourt, 1998; Grill and Himmelbach, 1998; Leung and Giraudat, 1998). In particular, genetic screens based on the inhibition of seed germination by applied ABA have led to the isolation of several Arabidopsis mutants with altered ABA responsiveness. Mutations in the ABA-insensitive (*ABI*) loci *ABI1* to *ABI5* reduce the sensitivity of seed germination to exogenous ABA (Koornneef et al., 1984; Ooms et al., 1993; Finkelstein, 1994a; Nambara et al., 1995). Conversely, mutations in the *ERA1* (*ENHANCED RESPONSE TO ABA*) locus increase the sensitivity of seed germination to applied ABA (Cutler et al., 1996).

The *abi3*, *abi4*, and *abi5* mutants exhibit additional defects in various aspects of seed maturation but do not seem to be altered in vegetative responses to ABA (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Ooms et al., 1993; Finkelstein, 1994a; Parcy et al., 1994; Nambara et al., 1995). The corresponding gene products thus may belong to a seed-specific branch of the ABA signaling network. The *ABI3* and *ABI4* genes have been cloned and encode putative transcriptional regulators (Giraudat et al., 1992; Finkelstein et al., 1998).

In contrast, the *abi1*, *abi2*, and *era1* mutations are more pleiotropic in that they affect ABA sensitivity in both seeds and vegetative tissues. The *ERA1* gene encodes the β subunit of a protein farnesyl transferase (Cutler et al., 1996). Loss-of-function *era1* mutants display prolonged seed dormancy and improved ability to withstand drought, which suggests that farnesylation is essential for negative regulation of ABA action (Cutler et al., 1996; Pei et al., 1998). The

[1] Current address: Biochimie et Physiologie Moléculaire des Plantes, Institut National de la Recherche Agronomique, Place Viala, 34060 Montpellier Cedex 1, France.

[2] Current address: Department of Plant Sciences, University of Cambridge, Downing St., Cambridge CB2 3EA, UK.

[3] To whom correspondence should be addressed. E-mail jerome.giraudat@isv.cnrs-gif.fr; fax 33-1-69-82-36-95.

ABI1 and *ABI2* genes encode homologous proteins that belong to the 2C class of protein serine/threonine phosphatases (PP2Cs; Leung et al., 1994, 1997; Meyer et al., 1994; Bertauche et al., 1996; Leube et al., 1998; Rodriguez et al., 1998a). The only mutant alleles available for these genes have been the *abi1-1* and *abi2-1* mutants isolated by Koornneef et al. (1984). These two mutants carry precisely the same amino acid substitution at equivalent positions in the ABI1 and ABI2 PP2C domains, respectively (Leung et al., 1997; Rodriguez et al., 1998a). The *abi1-1* and *abi2-1* mutations are both dominant and lead to largely overlapping sets of phenotypic alterations including ABA-resistant seed germination and seedling growth, reduced seed dormancy, abnormal stomatal regulation, and defects in various responses to drought stress (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Vartanian et al., 1994; Gosti et al., 1995; Leung et al., 1997).

The functions of the ABI1 and ABI2 phosphatases, however, could not be fully understood solely on the basis of the dominant mutant alleles identified thus far. First, it remained unclear whether these proteins actually are involved in ABA signaling. Indeed, it is possible that the wild-type ABI1 and ABI2 proteins do not normally contribute to ABA action and that only gain-of-function mutant forms can interfere with ABA responsiveness. Even if ABI1 and ABI2 are components of ABA signaling cascades, dominant mutant alleles do not permit us to conclude whether these proteins are positive or negative regulators of ABA responses. Furthermore, the possible contribution of PP2C proteins to ABA signaling cannot be easily investigated by pharmacological approaches because no potent inhibitor of this class of phosphatases has been identified (Hunter, 1995). It thus is required to characterize the phenotype of recessive loss-of-function mutants of these genes to elucidate the roles of ABI1 and ABI2 in ABA signal transduction.

In this study, we isolated seven distinct loss-of-function alleles of the *ABI1* gene as intragenic revertants of the *abi1-1* mutant. In marked contrast to the ABA-resistant *abi1-1* mutant, all of these intragenic revertants were more responsive to ABA than were wild-type plants, and this ABA supersensitivity phenotype was recessive. Moreover, each of these revertant alleles encodes an ABI1 protein that lacked any detectable phosphatase activity in an in vitro enzymatic assay. These results thus provide genetic evidence that a loss of ABI1 PP2C activity leads to an enhanced responsiveness to ABA, and hence that the wild-type ABI1 phosphatase is a negative regulator of ABA responses.

RESULTS

Isolation of Phenotypic Suppressors of *abi1-1*

Seeds homozygous for the *abi1-1* mutation were treated with ethyl methanesulfonate (EMS), and the M_2 population was screened for mutants showing a reversion of the ABA-resistant seed germination phenotype of *abi1-1*. M_2 seeds were plated along with wild-type and *abi1-1* controls on agar medium containing 3 μM ABA. After 4 days at 21°C, the wild-type seeds displayed either an intact seed coat or an emerged but growth-arrested seedling (Figure 1A), whereas in contrast, *abi1-1* seeds had germinated and produced seedlings with green expanded cotyledons and a well-developed root (Figure 1B). The M_2 seeds that resembled wild-type controls were selected and transferred onto ABA-free medium to rescue the viable individuals. The plantlets that developed well on ABA-free medium were retained as putative *abi1-1* revertants. These plantlets then were incu-

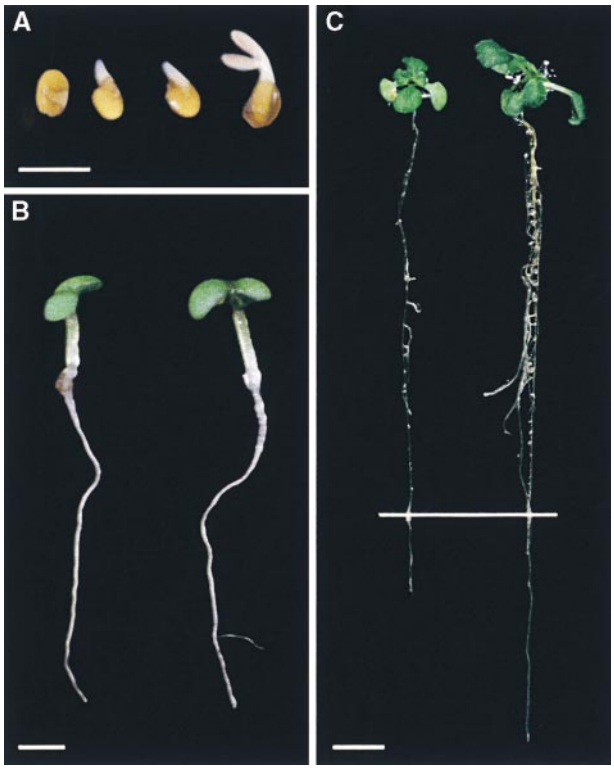

Figure 1. Phenotypic Criteria Used for the Mutant Screen.

(A) and **(B)** Sensitivity of seed germination to 3 μM ABA. Seeds of the L*er* wild type **(A)** and the *abi1-1* mutant **(B)** were plated on medium supplemented with 3 μM ABA, chilled for 3 days at 4°C in the dark, and incubated for 4 days at 21°C with a 16-hr-light photoperiod. Bars = 1 mm.

(C) Sensitivity of root growth to 10 μM ABA. Seedlings of the L*er* wild type (left) and the *abi1-1* mutant (right) were grown for 6 days on ABA-free medium and then incubated vertically for 3 days on an agar plate supplemented with 10 μM ABA. Upon transfer to the ABA-containing plate, the root tips of the seedlings were aligned on the white line. Hence, the portions of the roots that are located below the line were formed during incubation in the presence of ABA. Bar = 5 mm.

bated on agar medium containing 10 μM ABA, and their root growth after 3 days was compared with that of wild-type and *abi1-1* controls (Figure 1C). The candidate revertants selected in the germination assay then were divided into two classes, depending on whether they had regained wild-type ABA sensitivity for root growth.

From an estimated total of 4000 M_1 plants and 139,000 M_2 seeds screened, we finally retained 11 independent (originating from distinct pools of M_1 plants) lines that showed a significant reversion of *abi1-1* phenotypes in the M_3 generation. Three lines showed only partial reversion of the *abi1-1* phenotype in the germination and/or root growth assays of the screen. These three lines subsequently were shown to contain extragenic suppressor mutations, and their analysis will be described elsewhere. The eight other lines displayed a complete reversion of the ABA resistance of *abi1-1* in both the germination and the root growth assays used during the screen. These lines subsequently were found to be intragenic revertants (see below). Hence, as recommended by Meinke and Koornneef (1997), these mutants were designated as *abi1-1R1* to *abi1-1R8* to indicate that each of these novel alleles of *ABI1* contains the *abi1-1* mutation and a suppressor mutation (*R1* to *R8*). Because *abi1-1R5* and *abi1-1R8* contain exactly the same suppressor mutation (see below), only the *abi1-1R5* line was included in subsequent studies.

Table 1. Segregation of the Revertant Phenotype in an Homozygous *abi1-1* Background

Cross[a]	Generation	Phenotype[b] ABA Resistant	Phenotype[b] ABA Sensitive	χ^{2c}	P
L*er* × *abi1-1*	F_1	76	0		
	F_2	71	27	0.34	>0.6
abi1-1R1 × *abi1-1*	F_1	108	0		
	F_2	124	43	0.05	>0.8
abi1-1R2 × *abi1-1*	F_1	46	0		
	F_2	108	42	0.72	>0.4
abi1-1R3 × *abi1-1*	F_1	27	0		
	F_2	143	52	0.29	>0.6
abi1-1R4 × *abi1-1*	F_1	22	0		
	F_2	129	54	1.98	>0.15
abi1-1R5 × *abi1-1*	F_1	49	0		
	F_2	107	38	0.11	>0.7
abi1-1R6 × *abi1-1*	F_1	23	0		
	F_2	126	49	0.84	>0.3
abi1-1R7 × *abi1-1*	F_1	26	0		
	F_2	101	40	0.85	>0.3

[a] In each case, the F_1 seeds derived from the reciprocal cross were also all resistant to ABA (data not shown).
[b] Seeds were tested for their sensitivity to 3 μM ABA as described in Figure 1, and scored as ABA resistant or ABA sensitive by comparison to *abi1-1* and L*er* controls, respectively.
[c] Segregation data were evaluated with the chi-square goodness-of-fit test by using 3:1 segregation of the ABA-resistant phenotype as the null hypothesis. Chi-square values (χ^2) and corresponding probabilities (P) are indicated.

Genetic and Molecular Characterization of the Intragenic Revertants

Each of the *abi1-1R1* to *abi1-1R7* lines was backcrossed to *abi1-1.* In all cases, the resulting F_1 seeds were resistant to ABA, and the F_2 seed from self-fertilized F_1 plants showed 3:1 segregation for the ABA-resistant phenotype (Table 1). These results indicate that the suppressor mutations are nuclear, monogenic, and recessive to *abi1-1.*

To test linkage between the suppressor mutations and the *abi1-1* mutation, we outcrossed each of the *abi1-1R1* to *abi1-1R7* lines to wild-type Arabidopsis Landsberg *erecta* (L*er*). In the F_2 generation, individuals having inherited the *abi1-1* mutation but not the suppressor mutation would be expected to display the ABA-resistant phenotype of *abi1-1.* A sample (1 to 1.4 × 10^5 seeds) of each F_2 population was analyzed in the germination assay on 3 μM ABA, as described above. In all cases, the frequency of ABA-resistant individuals was lower than 0.01% (data not shown), indicating that the suppressor mutations are tightly linked to the *abi1-1* mutation. The ratio of physical to genetic distance in this region of chromosome 4 is ~150 kb per centimorgan (Leung et al., 1994; Schmidt et al., 1995), suggesting that the suppressor mutations were located within the *ABI1* gene itself.

The entire nucleotide sequence of the *ABI1* gene was determined from the *abi1-1R1* to *abi1-1R8* revertant lines. Each of these lines was found to contain the original *abi1-1* mutation together with an additional missense mutation within the *ABI1* open reading frame (Figure 2). Hence, combined genetic and molecular evidence indicates that these intragenic mutations are responsible for suppression of the *abi1-1* phenotype. The same suppressor mutation was found in the independent *abi1-1R5* and *abi1-R8* lines, whereas all the other suppressor mutations were isolated only once (in a single pool of M_1 plants).

All the intragenic suppressor mutations identified above translate into amino acid substitutions within the PP2C domain of the ABI1 protein (Figure 2). Remarkably, although these mutations are scattered along the primary structure of the ABI1 PP2C domain, they all occur within blocks of amino acids that are conserved in other PP2Cs (Pilgrim et al., 1995; Das et al., 1996) and are located in proximity to the catalytic site (Das et al., 1996).

The abi1-1R1 to abi1-1R7 Mutant Proteins Have No Detectable PP2C Activity in Vitro

The effect of the intragenic suppressor mutations on the PP2C activity of ABI1 was investigated using an in vitro

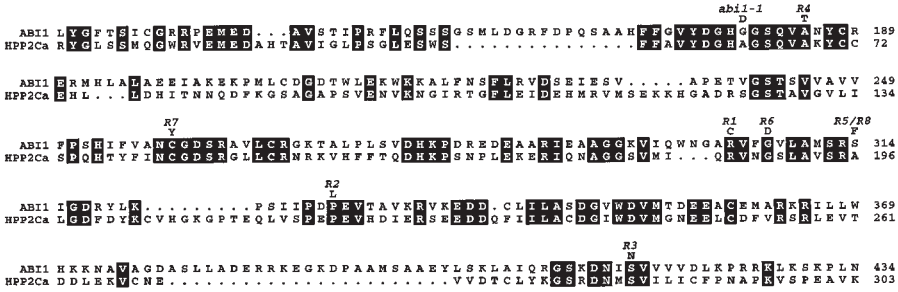

Figure 2. Localization of the Intragenic Suppressor Mutations within the Sequence of the PP2C Domain of ABI1.

Shown is an alignment of the amino acid sequences of the PP2C domain of ABI1 (ABI1) (Leung et al., 1994; Meyer et al., 1994) and of the human protein PP2Cα (HPP2Ca) (Mann et al., 1992). Residues conserved between ABI1 and HPP2Cα are displayed as white letters on a black background. Dots indicate gaps introduced to maximize alignment of conserved residues. The *abi1-1* mutation and the various suppressor mutations (*R1* to *R8*) are indicated above the ABI1 sequence. In the nucleotide sequence of the *ABI1* cDNA (GenBank accession number X77116), the suppressor mutations are G-to-A transitions at nucleotides 984 (*R4*), 1207 (*R7*), 1351 (*R6*), and 1678 (*R3*), and C-to-T transitions at nucleotides 1341 (*R1*), 1372 (*R5* and *R8*), and 1414 (*R2*).

phosphatase assay. Recombinant proteins that consisted of glutathione *S*-transferase (GST) fused to the wild type or various mutant forms of the ABI1 protein were produced in *Escherichia coli* and affinity purified. The amounts of fusion protein present in the various affinity-purified samples were standardized to each other by analysis on SDS–polyacrylamide gels (Figure 3A) and quantified by scanning the Coomassie blue–stained gel, using BSA as a standard. The recombinant proteins then were tested for their ability to dephosphorylate ^{32}P-labeled casein, an artificial substrate commonly used in assaying PP2C activity (MacKintosh, 1993).

When introduced alone (i.e., without the *abi1-1* mutation in the *ABI1* sequence), each of the four suppressor mutations tested engendered a severe reduction in the PP2C enzymatic activity of ABI1. The *R1* and *R6* mutations caused 60-fold decreases in the specific activity of the ABI1 protein, and the *R2* and *R5* mutations resulted in the loss of any detectable phosphatase activity in our experimental conditions (i.e., at least a 1000-fold reduction in specific activity; Table 2 and Figure 3B).

The suppressor mutations inhibited the ABI1 phosphatase activity also when they were combined with the *abi1-1* mutation. As previously described (Bertauche et al., 1996; Leube et al., 1998), the *abi1-1* mutation alone reduced the specific activity of the ABI1 phosphatase (Table 2). Remarkably, all the double mutant abi1-1R1 to abi1-1R7 proteins lacked any detectable phosphatase activity in our experimental conditions, indicating that their specific activities were at least 1000-fold lower than that of the wild-type ABI1 protein and 40-fold lower than that of the abi1-1 mutant protein (Table 2).

These results indicated that the intragenic suppressor mutations are likely loss-of-function mutations. Hence, it was of interest to analyze the effect of these mutations on ABA responsiveness. Because of the very tight genetic linkage between the suppressor mutations and the *abi1-1* mutation, it was difficult to isolate plants containing a suppressor mutation without the *abi1-1* mutation. As an alternative, we analyzed in more detail the ABA sensitivity of the *abi1-1R1* to *abi1-1R7* revertants. The in vitro enzymatic assay described above indicated that each of the *abi1-1R1* to *abi1-1R7* alleles encodes a catalytically inactive ABI1 protein, or at least an ABI1 protein with extremely reduced phosphatase activity. If the ABI1 phosphatase is not involved in ABA signaling, the ABA sensitivity of the loss-of-function *abi1-1R1* to *abi1-1R7* alleles should be identical to that of the wild type. Alternatively, the finding that these alleles differ from the wild type in their ABA sensitivity would demonstrate that the ABI1 phosphatase does regulate ABA signaling. Furthermore, a decrease in ABA sensitivity would indicate that ABI1 is a positive regulator of ABA action, whereas conversely, an increase in ABA sensitivity would imply that ABI1 is a negative regulator of ABA responses.

The Intragenic Revertants Have Enhanced Sensitivity to Applied ABA

The *abi1-1* mutant displays a lower sensitivity than the wild type to the inhibition of seed germination by exogenous ABA (Koornneef et al., 1984). Dose–response analyses showed that in agreement with the selection scheme used for mutant screening, this characteristic abi1-1 phenotype was suppressed in each of the *abi1-1R1* to *abi1-1R7* revertant alleles (Figure 4A and data not shown). Actually, as compared with the wild type, the intragenic revertants showed a phenotype opposite to that of *abi1-1*. Each of the *abi1-1R1* to *abi1-1R7* lines, indeed, was more sensitive than the wild type to the ABA inhibition of seed germination (Figure 4A and Table 3). In several independent experiments, the ABA dose required to achieve 50% inhibition of seed germination (ID_{50}) was approximately two- to threefold lower

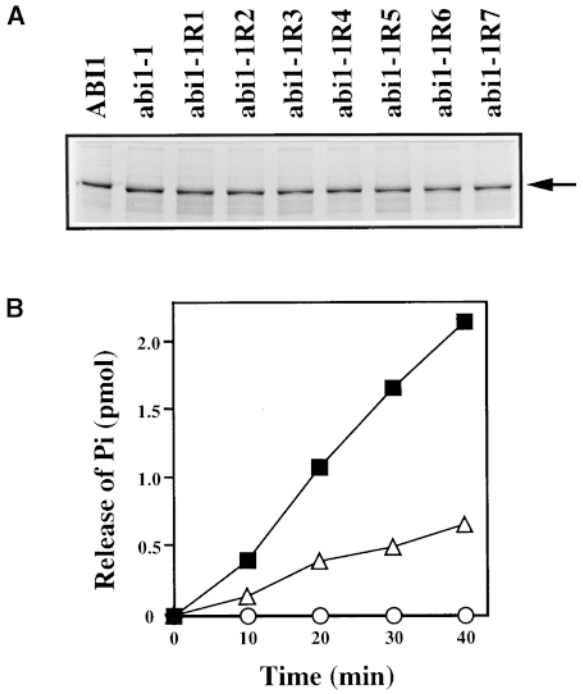

Figure 3. In Vitro PP2C Activity of Recombinant Proteins.

(A) Coomassie blue staining of a 10% SDS–polyacrylamide gel loaded with the indicated affinity-purified GST fusion proteins. The aliquots analyzed on the gel were estimated to contain 300 ng of intact fusion protein (indicated by the arrow) compared with BSA standards (not shown).

(B) In vitro protein phosphatase activity. GST–ABI1 (0.5 ng; filled squares), 15 ng of GST–abi1-R1 (open triangles), and 60 ng of GST–abi1-R2 (open circles) fusion proteins were incubated with ^{32}P-labeled casein at 30°C for the indicated times. Phosphatase activity is expressed in picomoles of ^{32}Pi released. Values have been subtracted for the amount of ^{32}Pi released in control samples that contained buffer only (always $<$0.1 pmol). Values shown are means of duplicate assays, and the range of values obtained was smaller than the symbol size.

for the revertants than for the wild type (Figure 4A and data not shown). Similarly, sensitivity of root growth to ABA inhibition was higher in each of the revertants than in the wild type (Figure 4B and Table 3). The ID_{50} for root growth inhibition was again approximately two- to threefold lower for the revertants (Figure 4B and data not shown). These results thus demonstrate that in these two bioassays, the seven distinct intragenic revertants all display a higher sensitivity to ABA than does the wild type.

If this increased ABA sensitivity actually is caused by the loss of ABI1 PP2C activity described above, one would expect the revertant alleles to be recessive to the wild-type *ABI1* allele. We thus examined the dominance relationships between the wild-type *ABI1* and the revertant *abi1-1R* alleles. Homozygous revertants were crossed to wild-type L*er* plants. The F_1 progeny and self-fertilized seed of each parent were tested for their ability to germinate in the presence of ABA. For each of the four independent revertants analyzed, the germination rate of the heterozygous F_1 progeny was not significantly different from that of wild-type L*er* seed (Table 4). These results indicate that each of the revertant alleles tested is recessive to the wild-type *ABI1* allele in enhancing the sensitivity of seed germination to applied ABA. These genetic data show that the increased ABA sensitivity of the revertant alleles is due to a loss of function in ABI1, and the data correlate well with the changes in ABI1 PP2C activity observed in vitro.

The Intragenic Revertants Have Enhanced Seed Dormancy and Drought Adaptive Responses

The dose–response analyses described above showed that the intragenic revertants are more sensitive than the wild type to applied ABA. We then investigated whether these mutants also display enhanced responsiveness to endogenous ABA under physiological conditions. Because ABA plays important roles in the promotion of seed dormancy and in the regulation of plant water status, we analyzed whether these processes are affected in the intragenic revertants.

The seeds of ABA-deficient mutants and of ABA-resistant mutants, including *abi1-1*, are less dormant than wild-type seeds (Karssen et al., 1983; Koornneef et al., 1984, 1989b; Léon-Kloosterziel et al., 1996). In contrast, each of the *abi1-1R1* to *abi1-1R7* revertants displayed an increased seed dormancy compared with the wild type (Table 3). This result indicates that the seeds of the revertants have an enhanced responsiveness to endogenous ABA.

Table 2. In Vitro Protein Phosphatase Activities

Protein	PP2C Activity[a] (pmol P_i released sec^{-1} mg^{-1})	Protein[b]	PP2C Activity (pmol P_i released sec^{-1} mg^{-1})
ABI1	1134	abi1-1	42
abi1-R1	15	abi1-1R1	$<$1
abi1-R2	$<$1	abi1-1R2	$<$1
abi1-R3	NT	abi1-1R3	$<$1
abi1-R4	NT	abi1-1R4	$<$1
abi1-R5	$<$1	abi1-1R5	$<$1
abi1-R6	19	abi1-1R6	$<$1
abi1-R7	NT	abi1-1R7	$<$1

[a] The specific phosphatase activities of the indicated GST fusion proteins were determined in vitro by using ^{32}P-labeled casein as substrate. Under our experimental conditions, the detection limit for PP2C activity was 1 pmol of Pi released per sec per mg of protein. NT, not tested.

[b] All the proteins indicated in this column contain the *abi1-1* mutation.

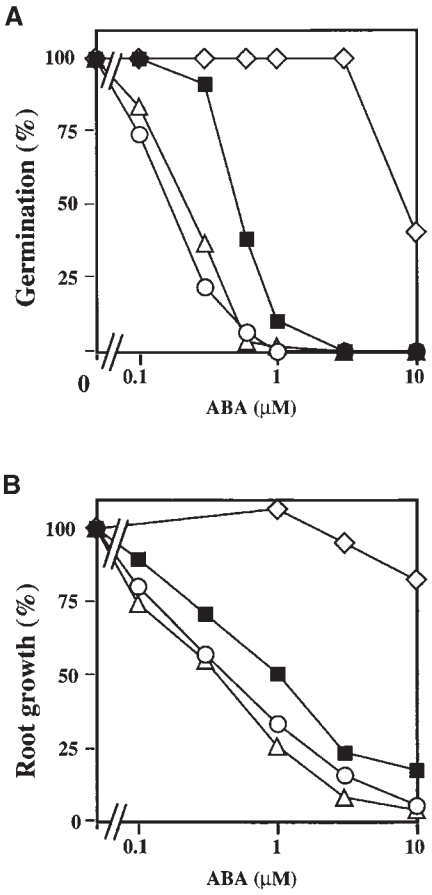

Figure 4. Sensitivity to Applied ABA.

(A) ABA dose response for germination inhibition. Seeds of L*er* wild type (filled squares), *abi1-1* (open diamonds), *abi1-1R1* (open circles), and *abi1-1R5* (open triangles) were plated on medium supplemented with the indicated concentrations of ABA, chilled for 4 days at 4°C in darkness, and incubated for 2 days at 21°C with a 16-hr-light photoperiod. The number of germinated seeds (with fully emerged radicle tip) was expressed as the percentage of the total number of seeds plated (100 to 200). For 0.1, 0.3, 0.6, and 1 μM ABA, the germination rates of the *abi1-1R1* and *abi1-1R5* revertants were lower than that of L*er* ($P < 0.001$; chi square test of independence).

(B) ABA dose response for root growth inhibition. Seeds of L*er* wild type (filled squares), *abi1-1* (open diamonds), *abi1-1R2* (open circles), and *abi1-1R3* (open triangles) were germinated and grown for 6 days on ABA-free medium. These seedlings then were incubated vertically on medium supplemented with the indicated concentrations of ABA, and their root growth was scored after 5 days. Root growth of ABA-treated seedlings is expressed as a percentage relative to controls incubated on ABA-free medium. Values shown are mean ±SE from samples composed of 20 to 30 seedlings each. In all cases, the error bars were smaller than the symbol size.

Another characteristic shared by the ABA-deficient and the *abi1-1* mutants is a defect in the regulation of stomatal aperture that leads to disturbed water relations (Koornneef et al., 1982, 1984; Léon-Kloosterziel et al., 1996). In isolated epidermal strips, stomata of *abi1-1* are more open than are those of the wild type (Leung et al., 1994; Roelfsema and Prins, 1995; Pei et al., 1997). In contrast, stomatal apertures were 15 to 20% smaller in the intragenic revertants tested (*abi1-1R1* and *abi1-1R5*) than in the wild type (e.g., 4.7 ± 0.1 μm in *abi1-1R1* versus 5.8 ± 0.1 μm; $n = 120$).

Water loss in excised leaves or aerial parts is markedly higher in *abi1-1* than in the wild type (Koornneef et al., 1984; Finkelstein, 1994b; Leung et al., 1997). The kinetics of water loss in detached leaves of each of the *abi1-1R1* to *abi1-1R7* revertants was similar to that in wild-type leaves (data not shown). The phenotype of the *abi1-1* mutant thus was fully suppressed in the intragenic revertants, but this assay on detached leaves did not permit us to distinguish the revertants from the wild type.

However, when whole plants were subjected to a progressive drought stress, the intragenic revertants tested (*abi1-1R1* and *abi1-1R3*) displayed an enhanced drought tolerance compared with the wild type, whereas in contrast, *abi1-1* was highly drought sensitive. When the soil water content had decreased to 35%, rosette leaves of *abi1-1* were permanently wilted, whereas wild-type plants only showed signs of withering (Figures 5A and 5B). When the soil water content had dropped further to 15%, the *abi1-1R3* plants still were flowering and had turgid rosette leaves, whereas wild-type plants already were completely wilted (Figures 5C and 5D).

Under these conditions of progressive drought stress, wild-type plants also develop an adaptive response at the root level, designated as drought rhizogenesis (Vartanian et al., 1994). This response results in the formation, from a threshold water deficit, of short, tuberized, and hairless roots that remain quiescent under prolonged drought stress but are able to rapidly recover elongation and hair formation upon rehydration. Endogenous ABA plays a promotive role in this process because the production of drought-induced short roots is reduced in the ABA-deficient *aba-1* mutant (Vartanian et al., 1994). Accordingly, the ABA-resistant *abi1-1* mutant also displayed a lower response than the wild type (Table 5; Vartanian et al., 1994). In contrast, the intensity of drought rhizogenesis was enhanced in the two revertants tested (*abi1-1R1* and *abi1-1R3*) relative to the wild type (Table 5). These results thus indicate that the intragenic revertants are more responsive to endogenous ABA levels under physiological conditions.

DISCUSSION

The only mutant allele of *ABI1* that has been analyzed thus far is the *abi1-1* mutant, which displays a marked reduction

Table 3. Phenotypic Analysis of Intragenic Revertants

Genotype	Seed Germination on 0.3 μM ABA[a] (%)	Seed Dormancy[b] (%)	Root Growth on 1 μM ABA[c] (%)	
			Experiment 1	Experiment 2
L*er*	90.9	53.6	50.9 ± 1.3	62.4 ± 1.1
abi1-1R1	21.5	0		44.2 ± 1.4
abi1-1R2	24.8	2.9	33.6 ± 2.0	
abi1-1R3	36.4	7.8	25.9 ± 1.2	
abi1-1R4	30.3	1.2	36.5 ± 0.9	
abi1-1R5	36.6	1.4		40.5 ± 1.6
abi1-1R6	41.8	0		46.9 ± 1.6
abi1-1R7	24.1	1.1	33.7 ± 1.7	

[a] Seeds were plated on medium containing 0.3 μM ABA, chilled for 4 days at 4°C in darkness, and incubated for 2 days at 21°C with a 16-hr-light photoperiod. The number of germinated seeds (with fully emerged radicle tip) was expressed as the percentage of the total number of seeds plated (100 to 200). All the revertants showed a lower germination rate than L*er* ($P < 0.001$, chi-square test of independence).

[b] Percentage of freshly harvested seeds that germinated after 6 days at 21°C (without cold pretreatment) on ABA-free medium (100 to 200 seeds tested). In parallel experiments in which seeds were first chilled for 4 days at 4°C in darkness to break dormancy, 100% germination was observed for all genotypes (data not shown).

[c] Seeds were germinated and grown for 6 days on ABA-free medium. These seedlings then were transferred to plates containing 1 μM ABA, and their root length was scored after 5 days. Root growth of ABA-treated seedlings is expressed as a percentage relative to controls incubated on ABA-free medium. The two columns derive from two separate experiments. Values shown are mean ±SE from samples composed of 20 to 30 seedlings each. All the revertants showed a lower root growth on ABA than L*er* ($P < 0.001$, Student's *t* test).

in ABA sensitivity. However, because the *abi1-1* mutation is dominant, it was impossible to conclude whether *ABI1* contributes to ABA signaling, and in case *ABI1* does regulate ABA responsiveness, whether it is a positive or a negative regulator of ABA action. We describe here the characterization of seven novel alleles of the *ABI1* gene (*abi1-1R1* to *abi1-1R7*). These mutants initially were selected as showing a reversion of the resistance of *abi1-1* to ABA inhibition of seed germination and seedling root growth. Actually, these mutants displayed a phenotype opposite that of *abi1-1*. Quantitative dose–response analyses revealed that in the two bioassays mentioned above, the *abi1-1R1* to *abi1-1R7* mutants are more sensitive to applied ABA than is the wild type. In addition, these mutants displayed increases in seed dormancy, whole-plant drought tolerance, and drought rhizogenesis intensity, which are indicative of increased responsiveness to the endogenous ABA levels that control these physiological processes.

The seven suppressor mutations (*R1* to *R7*) are missense mutations in conserved regions of the PP2C domain of ABI1. In the absence and in the presence of the *abi1-1* mutation, these suppressor mutations severely reduced the protein phosphatase activity of the ABI1 protein in our in vitro assay. In particular, the abi1-1R1 to abi1-1R7 proteins all lacked detectable PP2C activity, indicating that the specific activities of these mutant proteins was at least 1000-fold lower than that of the wild-type ABI1 protein. Moreover, the enhanced ABA sensitivities of the corresponding revertant plants appear to result from losses of function in *ABI1* because, in germination assays on ABA, the revertant alleles were recessive to both the *abi1-1* and the wild-type *ABI1* alleles. These combined genetic and molecular characteristics thus indicate that the ABA supersensitivity of the various revertants analyzed here is due to a loss of ABI1 phosphatase activity. It would be of clear interest to analyze the effect on ABA sensitivity of the suppressor mutations alone (i.e., in the absence of *abi1-1*), and currently, we are attempting to isolate such plants. However, the observation that the ABA-supersensitive phenotype of the revertant alleles is recessive to the wild-type *ABI1* allele, whereas the *abi1-1* mutation alone leads to a dominant ABA-resistance phenotype,

Table 4. Germination on ABA of Parental and F_1 Seeds

Genotype	Germinated/Tested[a]	%	P[b]
L*er*	117/122	95.9	
abi1-1R1	42/75	56.0	<0.001
F_1 (*abi1-1R1* × L*er*)	43/43	100.0	>0.15
L*er*	84/119	70.6	
abi1-1R3	18/88	20.4	<0.001
F_1 (*abi1-1R3* × L*er*)	19/24	79.2	>0.35
abi1-1R5	19/87	21.8	<0.001
F_1 (*abi1-1R5* × L*er*)	22/26	84.6	>0.15
abi1-1R6	28/114	24.6	<0.001
F_1 (*abi1-1R6* × L*er*)	25/31	80.6	>0.25

[a] Seeds were sown on medium with 0.6 μM ABA, chilled for 4 days at 4°C in darkness, and incubated for 2 days at 21°C with a 16-hr-light photoperiod. The *abi1-1R1* revertant was analyzed in an experiment separate from the other three.

[b] Probabilities derived from chi-square tests of independence.

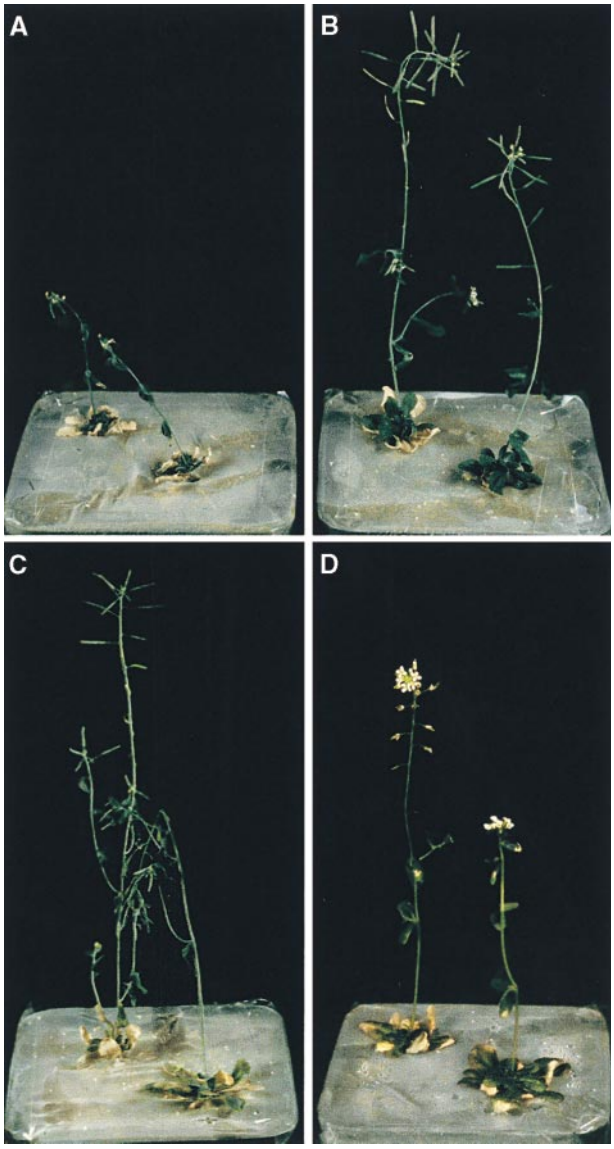

Figure 5. Differential Phenotypes of Plants Subjected to Progressive Drought Stress.

Plants were grown in a microphytotron under well-watered conditions for 1 month and then subjected to drought stress by withholding water. The soil surface was protected from evaporation by a sheet of Parafilm (American National Can, Chicago, IL).

(A) and **(B)** *abi1-1* and L*er* wild-type plants, respectively, were photographed when the soil water content had decreased to 35% (20 days after the onset of the progressive drought stress).

(C) and **(D)** L*er* wild-type and *abi1-1R3* plants, respectively, were photographed when the soil water content had decreased to 15%. This value of soil water content was reached 32 and 42 days after the onset of the drought stress for L*er* and *abi1-1R3*, respectively.

makes it unlikely that the *abi1-1R1* to *abi1-1R7* revertant alleles interfere with ABA sensitivity simply because of the presence of the *abi1-1* mutation. Hence, the present data indicate that a loss of ABI1 phosphatase activity leads to an enhanced responsiveness to ABA. This study thus provides genetic evidence that *ABI1* does regulate ABA sensitivity and, more specifically, that the ABI1 phosphatase is a negative regulator of ABA responses.

The ABA-supersensitive phenotype of the *abi1-1R1* to *abi1-1R7* revertants readily explains why recessive loss-of-function mutants of *ABI1* have not been isolated in screens based on ABA-resistant seed germination (Koornneef et al., 1984; Finkelstein, 1994a). Our results indicate that recessive mutants of *ABI1* should rather be identifiable in screens for seeds with increased sensitivity to the ABA inhibition of germination. Thus far, this type of screening process has led to the isolation of three classes of ABA-supersensitive mutants, which were designated as *era1* to *era3* (Cutler et al., 1996). The *era1* mutants have several phenotypes in common with the revertant alleles of *ABI1* described here (Cutler et al., 1996; Pei et al., 1998). However, the *ERA1* gene encodes the β subunit of a protein farnesyl transferase and thus clearly is distinct from *ABI1* (Cutler et al., 1996). Likewise, the *era3* mutation has been mapped to a chromosome other than *ABI1* (P. McCourt, personal communication). It remains to be investigated whether the *era2* mutant is allelic to *ABI1.*

Our screen clearly was not at saturation because only one suppressor mutation was isolated twice independently (in *abi1-1R5* and *abi1-1R8*). It is nevertheless intriguing that we did not recover any suppressor mutation introducing, for instance, a premature stop codon in the *ABI1* gene. Similarly, the five independent intragenic suppressors of *abi1-1* that were isolated by Steber et al. (1998) all contain missense mutations in the *ABI1* gene (F. Gosti, D. Guerrier, J. Giraudat, and P. McCourt, unpublished observations). It is thus likely that true molecular null alleles of *ABI1* were counterselected in the suppressor screens, although such alleles would be predicted to have lost the ABA-resistant phenotype of *abi1-1.* Among other possibilities, it is conceivable that molecular null alleles of *ABI1* might have a highly increased degree of seed dormancy and hence were not retained in the screens because of their poor germination rate, even in the absence of added ABA. In any case, characterizing the phenotype of knockout mutants would be instrumental in obtaining further insights into the physiological roles of *ABI1* in the plant. In principle at least, such mutants should be present as heterozygotes in collections of insertion mutants and can be sought for by molecular techniques.

As summarized above, this study provides genetic evidence that the phosphatase activity of the wild-type ABI1 protein negatively regulates ABA signaling. This conclusion is consistent with the recent report that constitutive overexpression of ABI1 inhibited ABA action in maize protoplasts (Sheen, 1998). The molecular details of ABI1 action in the plant, and in particular the nature of the endogenous sub-

Table 5. Drought Rhizogenesis

Genotype	Drought Rhizogenesis	Intensity[a]	P[b]
L*er*	13.48 ± 0.76	(*n* = 25)	
abi1-1	10.85 ± 0.65	(*n* = 23)	<0.02
abi1-1R1	17.12 ± 0.83	(*n* = 28)	<0.02
abi1-1R3	18.56 ± 1.71	(*n* = 22)	<0.02

[a] Number of short roots produced per milligram of total root biomass (dry matter weight). Mean ±SE (number of samples).
[b] Student's *t* test for differences between the indicated genotype and the L*er* wild type.

strate of this PP2C, are not known. Nevertheless, Figure 6 shows two simple models of the possible mode of action of ABI1 as a negative regulator of ABA signaling. These models postulate that the catalytic activity of the ABI1 phosphatase is not (Figure 6A) or is (Figure 6B) regulated by ABA, respectively. In the model in Figure 6A, the ABI1 protein belongs to a regulatory branch that attenuates ABA signaling. In the model in Figure 6B, ABI1 is a core element of the ABA transduction cascade, and ABA triggers physiological responses by inhibiting the catalytic activity of the ABI1 protein. Discriminating between these models most likely will require monitoring in the plant of the time course of the changes in ABI1 activity after the application of an ABA stimulus. The abundance of the *ABI1* mRNA is enhanced by ABA treatment, and this induction is inhibited in the *abi1-1* mutant (Leung et al., 1997). In both models discussed above, transcriptional induction of the *ABI1* gene thus could create a negative feedback regulatory loop. This loop might be responsible for resetting the ABA signaling cascade, thereby allowing the cell to continuously monitor the presence or absence of ABA. This scenario is analogous to the proposed role of other PP2Cs in the regulation of stress-induced pathways (Gaits et al., 1997; Meskiene et al., 1998).

The dominant *abi1-1* mutation converts Gly-180 to Asp in the vicinity of the PP2C active site (Leung et al., 1994; Meyer et al., 1994; Das et al., 1996) and leads to a decrease in ABA responsiveness (Koornneef et al., 1984). Our results indicate that the wild-type ABI1 phosphatase is a negative regulator of ABA signaling. Hence, a simple explanation of the *abi1-1* phenotype would be that this mutant protein has enhanced phosphatase activity. This possibility is, however, hard to reconcile with the observation that the mutant abi1-1 protein had a lower PP2C activity than did the wild-type ABI1 in both in vitro enzymatic assays and yeast mutant complementation studies (Bertauche et al., 1996; Leube et al., 1998). Nevertheless, in the model in Figure 6B, the phenotype of the *abi1-1* mutant could be explained by hypothesizing that the residual PP2C activity of this mutant protein is no longer regulated by ABA and that this constitutive activity is sufficient to inhibit ABA responsiveness. In the intragenic revertants, the loss of the residual PP2C activity of abi1-1 then would readily account for the suppression of the *abi1-1* phenotypes. Alternatively, in the model depicted in Figure 6A, one could hypothesize that the mutant abi1-1 protein has dominant negative effects because, as a result of conformational alterations in the catalytic site, it traps its endogenous substrate into a poison complex. This substrate then no longer would be able to play its role in relaying the ABA signal within the transduction cascade. The intragenic suppressor mutations, which all map to highly conserved domains in proximity to the ABI1 PP2C catalytic site, would disrupt the formation of the dysfunctional protein complex between abi1-1 and its substrate. The various hypotheses envisaged above can be experimentally tested with confidence only once the endogenous substrate of ABI1 has been identified.

The *ABI2* gene is closely related to *ABI1*. Furthermore, the dominant *abi1-1* and *abi2-1* mutations translate into precisely the same amino acid change in the PP2C domain of the corresponding protein and lead to largely similar phenotypes of

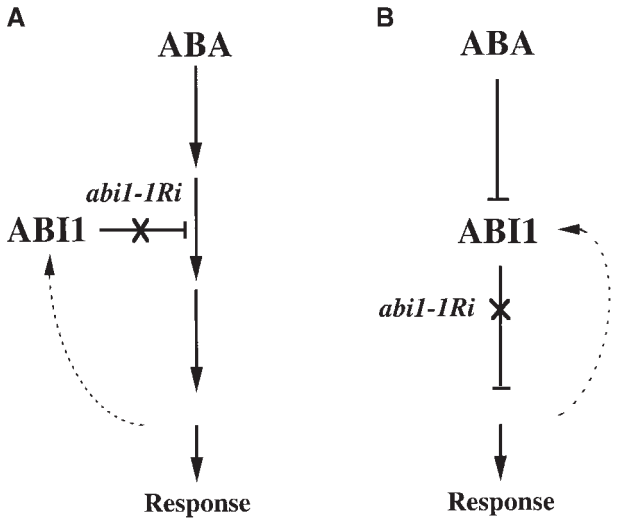

Figure 6. Hypothetical Models for the Role of ABI1 in ABA Signaling.

(A) The catalytic activity of the ABI1 protein is not regulated by ABA. Perception of the ABA signal stimulates the transduction cascade, leading to the activation of the physiological response. The ABI1 phosphatase attenuates the transduction cascade by dephosphorylating one of the signaling proteins. In the *abi1-1R1* to *abi1-1R7* revertant alleles (*abi1-1Ri*), the loss of ABI1 PP2C activity (indicated by the X) leads to enhanced ABA responsiveness. The ABA-induced elevation of *ABI1* mRNA amount creates a negative feedback loop (indicated by the dotted line).

(B) ABA inhibits the catalytic activity of the ABI1 protein. In the absence of ABA, the phosphatase activity of ABI1 represses the response. ABA (or an ABA-derived signal) inhibits the PP2C activity of ABI1, thereby releasing the repression and triggering the physiological response. In the revertant alleles, the loss of ABI1 PP2C activity (indicated by the X) enhances the response. The ABA-induced elevation of *ABI1* mRNA level creates a negative feedback loop (indicated by the dotted line).

ABA insensitivity (Koornneef et al., 1984; Leung et al., 1997; Rodriguez et al., 1998a). Our results thus suggest that by homology to ABI1, the wild-type ABI2 phosphatase as well would be a negative regulator of ABA action. These two homologous phosphatases are unlikely to have completely redundant roles because the revertant alleles of *ABI1* displayed a detectable ABA-supersensitive phenotype in various assays. Nevertheless, it would be informative to test whether simultaneous disruption of the ABI1 and ABI2 PP2C activities leads to a further increase in ABA responsiveness. Moreover, although corresponding mutants have not been isolated thus far, two additional Arabidopsis PP2Cs have been proposed to regulate ABA signal transduction (Rodriguez et al., 1998b; Sheen, 1998) and also may function as redundant negative regulators. The signaling pathways of various plant hormones recently have been shown to involve homologous and redundant negative regulators. ETR1-related ethylene receptors act as redundant negative regulators of ethylene responses in Arabidopsis (Hua and Meyerowitz, 1998). Likewise, available evidence suggests that the putative transcription factor *GAI* (for gibberellin insensitive) (Peng et al., 1997) and its homolog *RGA* (for repressor of the *gal-3* mutant (Silverstone et al., 1998) are redundant negative regulators of gibberellin action in Arabidopsis. Our findings suggest that ABA signaling also may be controlled by homologous and redundant negative regulators, in this case PP2Cs.

METHODS

Plant Material and Growth Conditions

The *abi1-1* line used in this work carries the linked visible marker *eceriferum4* (*cer4*) that was used to easily distinguish wild-type individuals (*CER*$^+$) from abscisic acid (ABA)–sensitive revertants derived from our mutagenized *abi1-1* population (*cer*$^-$). The recessive *cer4* mutation reduces the amount of epicuticular wax on the stem, which leads to a characteristic bright green color (Koornneef et al., 1989a). We verified that the *cer4* mutant does not significantly differ from the wild type in the various assays used in this study to analyze sensitivity to applied or endogenous ABA. For instance, seed germination rates were 88.4% for Landsberg *erecta* (L*er*) and 87.7% for *cer4* on 0.3 μM ABA, and 13.9% for L*er* and 18.3% for *cer4* on 0.6 μM ABA. Seedling root growth (expressed as a percentage relative to controls incubated on ABA-free medium) was 58.34 ± 0.98 for L*er* and 55.68 ± 1.27 for *cer4* on 0.6 μM ABA, and 10.54 ± 0.37 for L*er* and 11.83 ± 0.69 for *cer4* on 3 μM ABA. Water loss from detached aerial parts (expressed as a percentage of initial fresh weight) was 16.99 ± 1.39 for L*er* and 16.07 ± 3.06 for *cer4* after 20 min, and 22.64 ± 1.54 for L*er* and 21.57 ± 4.12 for *cer4* after 40 min. Stomatal apertures in isolated epidermal strips were 4.8 ± 0.1 μm (n = 120) for both L*er* and *cer4.* Finally, drought rhizogenesis intensity (number of short roots produced per milligram of total root biomass) was 13.48 ± 0.76 for L*er* and 13.24 ± 0.52 for *cer4.*

The *abi1-1* (Koornneef et al., 1984) and *cer4* (Koornneef et al., 1989a) mutants are both in the *Arabidopsis thaliana* ecotype L*er*, and the *abi1-1 cer4* double mutant was selected in the F_2 progeny of a cross between homozygous *abi1-1* and *cer4* single mutants.

Plants were routinely grown in a greenhouse (22°C with a 16-hr photoperiod) on soil irrigated with mineral nutrients. For aseptic growth, seeds were surface-sterilized and plated on a medium containing the inorganic salts of Murashige and Skoog (1962) at half concentrations, 100 mg/L myoinositol, 1 mg/L thiamine-HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 1% sucrose (unless otherwise indicated), 0.8% agar, and 2.5 mM (2-[*N*-morpholino] ethanesulfonic acid)–KOH, pH 5.7. ABA (mixed isomers; Sigma) was diluted from 10-mM stock solutions prepared in methanol; equivalent volumes of methanol were included in the ABA-free controls.

Mutagenesis and Mutant Screening

Approximately 4000 *abi1-1 cer4* seeds were mutagenized by imbibition in 0.4% (v/v) ethyl methanesulfonate (EMS; Sigma) for 8 hr at room temperature. After extensive washes with water to remove residual EMS, the mutagenized seeds (M_1) were sown and grown under standard greenhouse conditions. The M_2 seeds were harvested as 185 independent pools (each pool corresponding to ~15 viable M_1 plants).

To screen for revertants of *abi1-1*, we surface-sterilized ~750 seeds from each M_2 pool and plated the seeds on medium (without sucrose) supplemented with 3 μM ABA. Plates were kept in the dark at 4°C for 3 days to break seed dormancy and then were incubated at 21°C with a 16-hr-light photoperiod. After 4 days at 21°C, the seeds that displayed an appearance similar to that of wild-type controls (absence of radicle emergence or emerged-but-growth-arrested seedling; see Figure 1A) were transferred to agar plates without ABA. The seedlings that germinated and developed well after 7 days on the ABA-free medium were retained as candidate suppressors. These M_2 candidates then were examined for their sensitivity to the inhibition of root growth by ABA. The seedlings were incubated vertically on agar plates supplemented with 10 μM ABA, and their root growth was analyzed after 3 days. Finally, the selected plantlets were propagated in soil, and their phenotype was retested in the next (M_3) generation. Revertants originating from the same pool of M_2 seeds were assumed to be siblings, and only one representative was retained for further analysis.

Phenotypic Analyses

Detailed phenotypic characterization of the intragenic revertants was performed with lines that had been backcrossed at least once to *abi1-1 cer4.* Seeds used for comparative phenotypic studies were derived from maternal plants grown and harvested simultaneously. The kinetics of water loss from detached rosette leaves were analyzed as previously described (Leung et al., 1997). Stomatal aperture measurements on leaf epidermal strips were performed according to Webb and Hetherington (1997). To examine drought responses of whole plants, we subjected adult Arabidopsis to progressive drought stress as described previously (Vartanian et al., 1994). In brief, plants were grown for 1 month under well-watered conditions and then subjected to a progressive drought stress by withholding watering under controlled conditions in a microphytotron (temperature of 22°C, relative atmospheric humidity of 56%, and photon flux density of 200 μmol m^{-2} sec^{-1} during an 8-hr-light photoperiod). To determine the drought rhizogenesis intensity, we counted the number of

drought-induced short roots on plants harvested after the complete decline in the transpiration rate and when the soil moisture had decreased below 0.8% (Vartanian et al., 1994).

DNA Sequencing

Overlapping fragments encompassing the entire *ABI1* gene were obtained from plant genomic DNA (Konieczny and Ausubel, 1993) with polymerase chain reaction (PCR) by using specific primers. The amplified products were sequenced directly on both strands by using appropriate primers. Double-stranded DNA was sequenced on an Applied Biosystems (Foster City, CA) automated DNA sequencer (model 373A). Sequence analyses and alignments were performed using programs of the Genetics Computer Group (Madison, WI) software package (Devereux et al., 1984).

PP2C Activities

Recombinant proteins that consisted of glutathione *S*-transferase (GST) fused to the wild type or various mutant forms of the full-length ABI1 protein were produced in *Escherichia coli.* Nucleotides 432 to 794 of the *ABI1* cDNA (encoding the N-terminal part of ABI1) were amplified by PCR from pcABI1-C38 (GenBank accession number X77116; Leung et al., 1994) and cloned into the pGEX-2TK vector (Pharmacia Biotechnology) to create the pGEX-2TK/ABI1Nterm. The construct pGEX-2TK/ABI1 expressing a fusion between GST and the full-length wild-type ABI1 protein (GST–ABI1) then was generated by inserting into pGEX-2TK/ABI1Nterm the nucleotides 795 to 1926 of the *ABI1* cDNA recovered as an EcoRI fragment from pcABI1/Sma (Bertauche et al., 1996). The construct pGEX-2TK/abi1-1 expressing GST fused to the mutant abi1-1 protein (GST–abi1-1) was obtained by the same procedure, except that the mutant pcabi1-1/Sma plasmid (Bertauche et al., 1996) was used instead of pcABI1/Sma. Because the *R1* mutation destroys a unique SstI site in the *ABI1* gene, cDNAs corresponding to the *abi1-R1* and *abi1-1R1* mutant genes were created by site-directed mutagenesis of the *ABI1* and *abi1-1* cDNAs, respectively (Bertauche et al., 1996). Nucleotides 795 to 1926 of the *abi1-R1* and *abi1-1R1* cDNAs then were inserted into pGEX-2TK/ABI1Nterm to generate fusions between GST and the abi1-R1 (GST–abi1-R1) and abi1-1R1 (GST–abi1-1R1) proteins, respectively. cDNA fragments for the *abi1-1R2* to *abi1-1R7* mutant genes were generated by reverse transcription (RT)–PCR, starting from total RNA of 6-day-old plantlets that had been treated with 10 μM ABA for 3 days. For the *R2*, *R3*, *R5*, and *R6* suppressor mutations, the SstI-HindIII fragment comprising nucleotides 1341 to 1866 was excised from the RT-PCR product and cloned in place of the corresponding restriction fragment in pGEX-2TK/ABI1 or pGEX-2TK/abi1-1. For the *R4* and *R7* suppressor mutations, the BglII-SstI fragment comprising nucleotides 563 to 1341 was excised from the RT-PCR product and cloned in place of the corresponding restriction fragment in pGEX-2TK/abi1-1.

All constructs were verified by DNA sequencing. Recombinant proteins were expressed in the *E. coli* BL21 strain and were affinity purified on glutathione Sepharose 4B resin (Bertauche et al., 1996). Aliquots of the affinity-purified samples were analyzed by SDS-PAGE, and the amounts of recombinant proteins were quantified by scanning the Coomassie Brilliant Blue R 250–stained gel with BSA as a standard. Protein phosphatase activity was determined by standard procedures, using ^{32}P-labeled casein (0.5 to 0.9 $\times$ 10^9 cpm/μmol Pi) as substrate (MacKintosh, 1993; Bertauche et al., 1996). All reactions were performed in duplicate. Specific enzymatic activity was determined by measuring the velocity of ^{32}Pi release achieved with serial dilutions of the recombinant protein.

ACKNOWLEDGMENTS

We thank Maarten Koornneef for providing the *abi1-1* and *cer4* mutants; Camille Steber and Peter McCourt for sharing their intragenic revertants of *abi1-1*; Eric Requis for help during the initial characterization of *abi1-1R1*; Marcel Jacquin, Lucette Marcotte, and Rémy Drouen for technical assistance with plant culture; Liliane Troussard for technical assistance with DNA sequencing; and Nathalie Mansion for photographic work. We also thank Alistair Hetherington and Gerd Jürgens for stimulating discussions, and Jeff Leung, Sylvain Merlot, and François Parcy for their continuous interest and valuable comments on this manuscript. This work was supported in part by the Centre National de la Recherche Scientifique, the International Human Frontier Science Program (Grant No. RG-303/95), and the European Community BIOTECH program (Grant No. BIO4-CT96-0062).

Received July 9, 1999; accepted August 11, 1999.

REFERENCES

Bertauche, N., Leung, J., and Giraudat, J. (1996). Protein phosphatase activity of abscisic acid insensitive 1 (ABI1) protein from *Arabidopsis thaliana.* Eur. J. Biochem. **241,** 193–200.

Bonetta, D., and McCourt, P. (1998). Genetic analysis of ABA signal transduction pathways. Trends Plant Sci. **3,** 231–235.

Busk, P.K., and Pagès, M. (1997). Regulation of abscisic acid–induced transcription. Plant Mol. Biol. **37,** 425–435.

Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S., and McCourt, P. (1996). A protein farnesyl transferase involved in abscisic acid signal transduction in *Arabidopsis.* Science **273,** 1239–1241.

Das, A.K., Helps, N.R., Cohen, P.T.W., and Barford, D. (1996). Crystal structure of the protein serine/threonine phosphatase 2C at 2.0 Å resolution. EMBO J. **15,** 6798–6809.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12,** 387–395.

Finkelstein, R.R. (1994a). Mutations at two new *Arabidopsis* ABA response loci are similar to the *abi3* mutations. Plant J. **5,** 765–771.

Finkelstein, R.R. (1994b). Maternal effects govern variable dominance of two abscisic acid response mutations in *Arabidopsis thaliana.* Plant Physiol. **105,** 1203–1208.

Finkelstein, R.R., and Somerville, C.R. (1990). Three classes of abscisic acid (ABA)–insensitive mutations of *Arabidopsis* define genes that control overlapping subsets of ABA responses. Plant Physiol. **94,** 1172–1179.

Finkelstein, R.R., Wang, M.L., Lynch, T.J., Rao, S., and Goodman, H.M. (1998). The Arabidopsis abscisic acid response locus *ABI4* encodes an APETALA2 domain protein. Plant Cell **10,** 1043–1054.

Gaits, F., Shiozaki, K., and Russell, P. (1997). Protein phosphatase 2C acts independently of stress-activated kinase cascade to regulate the stress response in fission yeast. J. Biol. Chem. **272,** 17873–17879.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F., and Goodman, H.M. (1992). Isolation of the Arabidopsis *ABI3* gene by positional cloning. Plant Cell **4,** 1251–1261.

Gosti, F., Bertauche, N., Vartanian, N., and Giraudat, J. (1995). Abscisic acid–dependent and –independent regulation of gene expression by progressive drought in *Arabidopsis thaliana.* Mol. Gen. Genet. **246,** 10–18.

Grill, E., and Himmelbach, A. (1998). ABA signal transduction. Curr. Opin. Plant Biol. **1,** 412–418.

Hua, J., and Meyerowitz, E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana.* Cell **94,** 261–271.

Hunter, T. (1995). Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. Cell **80,** 225–236.

Karssen, C.M., Brinkhorst-van der Swan, D.L.C., Breekland, A.E., and Koornneef, M. (1983). Induction of dormancy during seed development by endogenous abscisic acid: Studies on abscisic acid deficient genotypes of *Arabidopsis thaliana* (L.) Heynh. Planta **157,** 158–165.

Konieczny, A., and Ausubel, F.M. (1993). A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J. **4,** 403–410.

Koornneef, M., Jorna, M.L., Brinkhorst-van der Swan, D.L.C., and Karssen, C.M. (1982). The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heynh. Theor. Appl. Genet. **61,** 385–393.

Koornneef, M., Reuling, G., and Karssen, C.M. (1984). The isolation and characterization of abscisic acid–insensitive mutants of *Arabidopsis thaliana.* Physiol. Plant. **61,** 377–383.

Koornneef, M., Hanhart, C.J., and Thiel, F. (1989a). A genetic and phenotypic description of *eceriferum* (*cer*) mutants in *Arabidopsis thaliana.* J. Hered. **80,** 118–122.

Koornneef, M., Hanhart, C.J., Hilhorst, H.W.M., and Karssen, C.M. (1989b). In vivo inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana.* Plant Physiol. **90,** 463–469.

Léon-Kloosterziel, K.M., Alvarez Gil, M., Ruijs, G.J., Jacobsen, S.E., Olszewski, N.E., Schwartz, S.H., Zeevaart, J.A.D., and Koornneef, M. (1996). Isolation and characterization of abscisic acid–deficient *Arabidopsis* mutants at two new loci. Plant J. **10,** 655–661.

Leube, M.P., Grill, E., and Amrhein, N. (1998). ABI1 of *Arabidopsis* is a protein serine/threonine phosphatase highly regulated by the proton and magnesium ion concentration. FEBS Lett. **424,** 100–104.

Leung, J., and Giraudat, J. (1998). Abscisic acid signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. **49,** 199–222.

Leung, J., Bouvier-Durand, M., Morris, P.-C., Guerrier, D., Chefdor, F., and Giraudat, J. (1994). *Arabidopsis* ABA-response gene *ABI1*: Features of a calcium-modulated protein phosphatase. Science **264,** 1448–1452.

Leung, J., Merlot, S., and Giraudat, J. (1997). The Arabidopsis *ABSCISIC ACID–INSENSITIVE2* (*ABI2*) and *ABI1* genes encode redundant protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell **9,** 759–771.

MacKintosh, C. (1993). Assay and purification of protein (serine/threonine) phosphatases. In Protein Phosphorylation: A Practical Approach, D.G. Hardie, ed (Oxford, UK: Oxford University Press), pp. 197–230.

Mann, D.J., Campbell, D.G., McGowan, C.H., and Cohen, P.T. (1992). Mammalian protein serine/threonine phosphatase 2C: cDNA cloning and comparative analysis of amino acid sequences. Biochim. Biophys. Acta **1130,** 100–104.

McCarty, D.R. (1995). Genetic control and integration of maturation and germination pathways in seed development. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46,** 71–93.

Meinke, D., and Koornneef, M. (1997). Community standards for *Arabidopsis* genetics. Plant J. **12,** 247–253.

Meskiene, I., Bögre, L., Glaser, W., Balog, J., Brandstötter, M., Zwerger, K., Ammerer, G., and Hirt, H. (1998). MP2C, a plant protein phosphatase 2C, functions as a negative regulator of mitogen-activated protein kinase pathways in yeast and plants. Proc. Natl. Acad. Sci. USA **95,** 1938–1943.

Meyer, K., Leube, M.P., and Grill, E. (1994). A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana.* Science **264,** 1452–1455.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 493–497.

Nambara, E., Keith, K., McCourt, P., and Naito, S. (1995). A regulatory role for the *ABI3* gene in the establishment of embryo maturation in *Arabidopsis thaliana.* Development **121,** 629–636.

Ooms, J.J.J., Léon-Kloosterziel, K.M., Bartels, D., Koornneef, M., and Karssen, C.M. (1993). Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana.* A comparative study using abscisic acid–insensitive *abi3* mutants. Plant Physiol. **102,** 1185–1191.

Parcy, F., Valon, C., Raynal, M., Gaubier-Comella, P., Delseny, M., and Giraudat, J. (1994). Regulation of gene expression programs during Arabidopsis seed development: Roles of the *ABI3* locus and of endogenous abscisic acid. Plant Cell **6,** 1567–1582.

Pei, Z.-M., Kuchitsu, K., Ward, J.M., Schwarz, M., and Schroeder, J.I. (1997). Differential abscisic acid regulation of guard cell slow anion channels in Arabidopsis wild-type and *abi1* and *abi2* mutants. Plant Cell **9,** 409–423.

Pei, Z.-M., Ghassemian, M., Kwak, C.M., McCourt, P., and Schroeder, J.I. (1998). Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. Science **282,** 287–290.

Peng, J., Carol, P., Richards, D.E., King, K.E., Cowling, R.J., Murphy, G.P., and Harberd, N.P. (1997). The *Arabidopsis GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. Genes Dev. **11,** 3194–3205.

Pilgrim, D., McGregor, A., Jäckle, P., Johnson, T., and Hansen, D. (1995). The *C. elegans* sex-determining gene *fem-2* encodes a putative protein phosphatase. Mol. Biol. Cell **6,** 1159–1171.

Rock, C.D., and Quatrano, R.S. (1995). The role of hormones during seed development. In Plant Hormones, P.J. Davies, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 671–697.

Rodriguez, P.L., Benning, G., and Grill, E. (1998a). ABI2, a second protein phosphatase 2C involved in abscisic acid signal transduction in *Arabidopsis.* FEBS Lett. **421,** 185–190.

Rodriguez, P.L., Leube, M.P., and Grill, E. (1998b). Molecular cloning in *Arabidopsis thaliana* of a new protein phosphatase 2C (PP2C) with homology to ABI1 and ABI2. Plant Mol. Biol. **38,** 879–883.

Roelfsema, M.R.G., and Prins, H.B.A. (1995). Effect of abscisic acid on stomatal opening in isolated epidermal strips of *abi* mutants of *Arabidopsis thaliana.* Physiol. Plant. **95,** 373–378.

Schmidt, R., West, J., Love, K., Lenehan, Z., Lister, C., Thompson, H., Bouchez, D., and Dean, C. (1995). Physical map and organization of *Arabidopsis thaliana* chromosome 4. Science **270,** 480–483.

Sheen, J. (1998). Mutational analysis of protein phosphatase 2C involved in abscisic acid signal transduction in higher plants. Proc. Natl. Acad. Sci. USA **95,** 975–980.

Silverstone, A.L., Ciampaglio, C.N., and Sun, T.-p. (1998). The Arabidopsis *RGA* gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. Plant Cell **10,** 155–169.

Steber, C.M., Cooney, S.E., and McCourt, P. (1998). Isolation of the GA-response mutant *sly1* as a suppressor of *ABI1-1* in *Arabidopsis thaliana.* Genetics **149,** 509–521.

Vartanian, N., Marcotte, L., and Giraudat, J. (1994). Drought rhizogenesis in *Arabidopsis thaliana.* Differential responses of hormonal mutants. Plant Physiol. **104,** 761–767.

Webb, A.A.R., and Hetherington, A.M. (1997). Convergence of the abscisic acid, CO_2, and extracellular calcium signal transduction pathways in stomatal guard cells. Plant Physiol. **114,** 1557–1560.

Zeevaart, J.A.D., and Creelman, R.A. (1988). Metabolism and physiology of abscisic acid. Annu. Rev. Plant Physiol. Plant Mol. Biol. **39,** 439–473.